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PATENT

ENDOTHELIAL CELL GROWTH FACTOR
AND METHODS OF ISOLATION

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BACKGROUND OF INVENTION

The present application is a continuation-in-part of pending U.S. Patent Application Serial No. 328,181, filed March 24, 1989, which is incorporated by reference in its entirety.

5

Origin of the Invention

The present invention was made with Government support in part on U.S. National Institute of Health Grant Nos. 5ROIEY 02186; 5ROIHL 20197; and HD 08035 awarded by the 10 Department of Health and Human Services to the University of California at San Francisco, California. The U.S. Government has certain rights in this invention.

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Field of the Invention

The present invention relates to a novel growth factor for vascular endothelial cells identified in media conditions by bovine pituitary follicular cells. The invention also related to isolation and purification of the growth factor.

20

Description of the Problem and Related Art

Numerical references in parenthesis in the text refer to the publications listed below in the Reference Section.

25

Angiogenesis is a multi-step phenomenon which involves capillary endothelial cell proliferation, migration and tissue infiltration (1). It plays a central role in a variety of physiological and pathological processes such as

embryonic development, wound healing, atherosclerosis and tumor growth (1,2). Several factors induce angiogenesis have recently been isolated and characterized. Among these are the acidic and basic form of fibroblast growth factor (FGF), both capable of stimulating capillary endothelial cell growth in vitro as well as being chemotactic for that cell type (2). In addition, both acidic and basic FGF stimulate collagenase activity and plasminogen activator production while blocking the activity of plasminogen inhibitor (3,4). These enzymes are involved in the breakdown of the capillary basement membrane, an event required in order for angiogenesis to take place (1). Other growth factors such as tumor necrosis factor alpha (TGF α), transforming growth factor beta (TGF β), transforming growth factor alpha (TGF α), and epidermal growth factor (EGF) are also angiogenic in vivo (5-8). However, with the exception of TGF α and EGF at high concentrations (7), these growth factors are not mitogenic for capillary endothelial cells (5,6); their action on the angiogenic process is therefore probably indirect, resulting from such activities as the attraction of macrophages by chemotaxis (9,10) which in turn release direct angiogenic factor(s), one of which could be basic FGF (11).

A number of growth factors, such as acidic and basic FGF, PDGF and EGF, are broadly mitogenic for a number of cell types. This broad mitogenicity is desirable in many types of wound healing applications. There are, however,

specific types of wound healing applications in which it would be more desirable to employ growth factors having more cell-specific mitogenic activity. For example, following vascular graft surgery or balloon angioplasty, it would be highly desirable to employ a wound healing agent incorporating mitogenic factor having mitogenic activity that is highly specific for vascular endothelial cells. At present, no highly suitable mitogenic factor exists for this type of application.

10 In the course of our studies on the localization of basic FGF in various tissues, it was observed that, in the pituitary gland, folliculo stellate cells are the main producers of bFGF (18). Although the medium conditioned by those cells was found to be strongly mitogenic for 15 capillary endothelial cells, little if any bFGF is present in it, thus suggesting that, in addition to synthesizing bFGF, these cells are also capable of producing another endothelial cell mitogen. To date, however, this mitogenic activity has not been purified or characterized.

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All of the references and/or patents cited in this application are incorporated herein by reference.

Previously published research describes the culture of homogeneous populations of bovine pituitary follicular or 5 folliculo-stellate cells (FC) (20) and subsequently characterized them as ion transport elements, possibly involved in the regulation of ion composition and osmolarity of the interstitial fluid in the adenohypophyseal cell cords (41, 42). It is also reported 10 that FC produce the angiogenic mitogen basic fibroblast growth factor (bFGF) (17).

The gene for bFGF (43), similarly to the gene for acidic fibroblast growth factor (aFGF) (13), does not code for a conventional signal peptide, required for the 15 extracellular transport of proteins according to classic secretory pathways (44). Accordingly, the growth factor is not appreciably secreted in the medium (15,45) and responsive cell types are dependent on exogenous bFGF for optimal proliferation in culture, even though they may 20 contain significant intracellular concentrations of mitogen (46,47,48).

It was initially observed, however, that the medium conditioned by bovine pituitary FC is mitogenic for adrenal-cortex-derived capillary endothelial cells. 25 Interestingly, these cells are responsive either to bFGF or aFGF but are not stimulated to proliferate by EGF, TGF alfa, TGF beta, PDGF, insulin or TNF (2). These observations led us to consider the possibility that an

endothelial cell growth factor distinct from FGF and possibly any other known growth factor may be secreted by cultured FC.

5 The present invention describes the purification and biological characterizations of such novel growth factor. Its unique N-terminal amino acid sequence, as well as its specificity for vascular endothelial cells, distinguishes it from any previously described growth factor.

SUMMARY OF THE INVENTION

10 In accordance with the present invention, there is provided a novel growth factor in isolated form, that is, unaccompanied by impurities which normally accompany the native molecule when it is produced *in vivo*. The growth factor of the invention shall be referred to herein as
15 "folliculo stellate-derived endothelial cell growth factor" (FSdGF), since it was originally isolated from bovine folliculo stellate cells or as "vascular endothelial growth factor" (VEGF). It is to be understood, however, that these terms are intended herein to encompass the protein,
20 regardless of its source or manner of production. For example, the provision herein of FSdGF in isolated form provides the means for isolating cloned DNA sequences encoding the protein, so that it can be produced in commercial quantities using the known techniques of
25 recombinant DNA technology. Furthermore, FSdGF herein is intended to encompass the corresponding proteins produced by other than bovine species, e.g. the human protein, even though it is known that minor variations in amino acid

sequence from species to species may occur which do not significant affect the useful activities of a protein. Using materials and procedures described herein those skilled in the art can obtain, for example, the 5 corresponding human protein by isolating and expressing cloned DNA sequences encoding the protein. Also included within the scope of the term "FSdGF" herein are biologically active fragments thereof, as well as N-terminally and/or C-terminally extended versions thereof 10 which retain qualitatively the biological activities of the FSdGF described herein. While the form of FSdGF which was isolated using procedures described herein is apparently glycosylated, it is known that production of proteins by recombinant means in certain procaryotic hosts such as E. 15 coli generally does not result in glycosylated forms of the protein, but that the resulting unglycosylated forms are often quite useful. Accordingly, the term "FSdGF" encompasses glycosylate and unglycosylated forms of the molecule, provided that they retain qualitatively the 20 biological activities described herein.

FSdGF is a dimeric protein of approximately 43 kd, as determined by SDS polyacrylamide gel electrophoresis under reducing conditions. It appears to exhibit cell-specific mitogenic activity on vascular endothelial cells. 25 Consequently, FSdGF will find use as a growth factor in a variety of wound healing applications in which it is desired to promote re-endothelialization in the vascular system. FSdGF will be particularly useful as a post-

operative wound healing agent in both vascular graft surgery and balloon angioplasty. FSdGF can also be employed as a mitogenic agent for growing endothelial cells in vitro. Yet another application for FSdGF is the 5 promotion of vascular wound healing following myocardial infarction.

In accordance with the invention, FSdGF can be obtained in isolated form from conditioned cell culture media containing FSdGF by a process which includes the 10 steps of ammonium sulfate precipitation; heparin sepharose affinity chromatography; exclusion gel chromatography; cation exchange chromatography; and reverse phase high pressure liquid chromatography.

BRIEF DESCRIPTION OF THE FIGURES

15 Figure 1A shows the heparin sepharose affinity chromatography (HSAC) of the conditional medium by FS cells.

Figure 1B shows the gel exclusion chromatography of the partially purified HSAC fractions on BioGel P-60.

20 Figure 1C shows the chromatographic results on Mono S of the bioactive BioGel P-60 fraction.

Figure 2A shows the reverse phase HPLC of the Mono S purified and bioactive fractions, and a comparison of the ability of the FS cell conditioned medium at various stages 25 of purification to stimulate the proliferation of low density ACE cells cultures.

Figure 2B is a comparison of the ability of FS cell conditioned medium at various stages of purification to

stimulate the proliferation of low density ACE cell cultures.

Figure 3 is the NaDODSO₄/PAGE of the bioactive fraction purified by RP C₄ HPLC.

5 Figure 4A, 4B and 4C are a comparison between the ability of pituitary derived bFGF versus FSdGF to stimulate growth of HUE cells (A), ACE cells (B) and BHK-21 cells (C).

10 Figure 5 is a comparison of the ability of bFGF versus FSdGF to stimulate the proliferation of BCE cells, granulosa cells, adrenal cortex cells and BALB/MK cells.

Figure 6 is a spectrum of reduced/alkylated/lysine blocked FEGF.

15 Figure 7 is a trace of the trypsin digestion of the growth factor.

Figure 8 is a graph of the reversed-phase high performance liquid chromatography of follicular cells mitogen activity.

20 Figure 9A and 9B are a NadoSO₄/PAGE (12.5 acrylamide) analysis of the most bioactive fraction from the previously shown HPLC profile.

Figure 10 shows a graph of the proliferation of the low density adrenal cortex derived endothelial cells as a function of time.

25 Figure 11 (as 11A, 11B and 11C) shows three photographs of the morphological appearance of low density adrenal cortex endothelial cells.

Figure 12 is a plot of the heparin-sepharose

bioactivity profile of a folliculo cell conditioned medium.

Detailed Description of the Invention

As used herein, the following abbreviations have the indicated meanings:

5	aFGF	acidic fibroblast growth factor
	bFGF	basic fibroblast growth factor
	PDGF	platelet derived growth factor
	TGF α	transforming growth factor α
	TGF β	transforming growth factor β
10	EGF	epidermal growth factor
	PDEC GF	platelet derived endothelial cell growth factor
	FS	folliculo stellate cells
	FSdGF	folliculo stellate derived growth factor
15	STV	0.01 M sodium phosphate (pH 7.4), 0.9% NaCl, 0.05% trypsin, 0.02% EDTA
	CS	calf serum
	FCS	fetal calf serum
	PBS	phosphate buffered saline
20	HSAC	heparin sepharose affinity chromatography
	RP-HPLC	reverse phase high pressure liquid chromatography
	FPLC	fast high pressure liquid chromatography
	ACE cells	adrenal cortex-derived capillary endothelial cells
25	HUE cells	human umbilical endothelial cells
	BCE cells	bovine corneal endothelial cells
	RIA	radioimmunoassay

BSA bovine serum albumin
BHK21 baby hamster kidney-derived fibroblast
 clone 21
Na Dod SO₄ sodium dodecyl sulfate
5 PAGE polyacrylamide gel electrophoresis
MW molecular weight
kDa kilo Dalton
DMEM Dulbecco's modified Eagle's medium

Materials

10 Bio Gel P-60, Bio Rad protein assay kit, silver nitrate stain kit and low molecular weight standards for Na Dod SO₄/PAGE were from Bio Rad (Richmond, CA). Heparin Sepharose, Concanavalin A Sepharose, and Mono S column HR5/5 were obtained from Pharmacia (Piscataway, NJ). The
15 Vydac C₄ reverse phase column was purchased from Separation Group (Hesperia, CA). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Grand Island Biological Co. (Grand Island, NY). STV (saline containing 0.05% Trypsin, 0.01 M sodium phosphate pH 7.3 and 0.02% EDTA) was obtained from
20 Difco Lab (Detroit, MI). Calf serum (CS) and fetal calf serum (FCS) were obtained by HyClone Sterile Systems, Inc. (Logan, UT). Tissue culture dishes were purchased from Falcon Plastics (Oxnard, CA), except for large scale Nunc culture plates (600 cm²) which were from Applied Scientific (San Francisco, CA). Gentamicin was obtained from Schering Co. (Kenilworth, NJ), and Fungizone was purchased from E.R. Squibb and Sons (Princeton, NJ). Leupeptin, gelatin,

transferring and insulin were Sigma (St. Louis, MO). Pituitary derived basic FGF and neutralizing rabbit polyclonal antibodies directed against basic FGF were prepared as previously described (18,19).

5 Cell culture

Pituitary derived folliculo stellate (FS) cell cultures were prepared and characterized as previously described (17,20). Confluent cultures, which consisted of homogeneous dome-forming cell monolayers, were dissociated 10 by exposure to STV supplemented with Na₂EDTA to a final concentration of 0.3% (4-5 min, 24°C). The cells were then seeded at a split ratio of 1:10 into large-scale culture plates and grown in the presence of DMEM supplemented with 5% CS, 5% FSC, 50 µg/ml gentamicin and 2.5 µg/ml Fungizone 15 (18). Upon reach confluency, cultures were further passaged or exposed to serum free medium (see below). Cultures of human umbilical endothelial cells (21), bovine brain and adrenal cortex derived capillary endothelial cells (22), bovine granulosa cells (23), adrenal cortex 20 cells (24), corneal endothelial cells (25), baby hamster kidney cells clone 21 (BHK-21)(26), and BALB/MK mouse epidermal keratinocytes (27) (a gift from Dr. S. Aaronson, NIH NCI, Bethesda, MD) were maintained as previously described (21-27).

25 Preparation of condition medium

Early passage FS cells were plated onto 600 cm² Nunc plates and grown to confluence over 4 to 5 days in DMEM supplemented with 5% CS, 5% FCS and antibodies as described

above. Once domes formation was observed, the monolayers were washed twice with 25 ml of phosphate buffered saline prior to the addition of 150 ml per plate of DMEM supplemented with 50 μ g/ml gentamicin, 25 μ g/ml Fungizone, 5 10 μ g/ml leupeptin, 5 μ g/ml insulin and 10 μ g/ml transferrin. After 48 or 72 hr, culture fluids were collected and replaced with the same amount of fresh serum free medium. Collections could be made for a month without visible deterioration of the monolayer.

10 Isolation procedure

Conditioned medium collected from the confluent monolayers was centrifuged (10,000g, 15 min) in order to remove floating cells and cell debris. The pH of the supernatant was then adjusted to 5.6 with 6N HCl. Ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$ (520 g/liter) was added, and the suspension was set for 6 hr 4°C, the precipitate was then collected by centrifugation (10,000g, 30 min), redissolved in PBS, and stored at -70°C.

For final isolation, the precipitates from 3 collections (21 liters total of conditioned medium, starting material) were thawed, pooled and then dialyzed overnight at 4°C against 10m mM Tris-HCl pH 7.3, 50 mM NaCl. Following dialysis the insoluble material was removed by centrifugation (10,000 g, 30 min) and the supernatant was loaded onto a heparin Sepharose resin (20ml) that had been equilibrated in 10 mM Tris-HCl pH 7.3, 50 mM NaCl. The resin was washed extensively with the equilibration buffer until the absorbance had returned to

baseline, and was then eluted stepwise with increasing NaCl concentrations (0.15 M, 0.45 M, 1 M and 3M NaCl). Aliquots were removed from the fractions for cell proliferation assays, and fractions with the highest 5 bioactivity were pooled and concentrated to 1 ml with an Amicon ultrafiltration cell (Model 12) equipped with a Diaflo YM 10 ultrafiltration membrane.

The concentrated sample was loaded onto a Bio Gel P-60 column (100-200 mesh 1 x 95 cm) equilibrated at 4°C in PBS 10 and was eluted with PBS. The Bio Gel P-60 column may be replaced with a Sephadex G-100 which appears to be more efficient. Aliquots of each fraction were taken for cell proliferation assay and the bioactive fractions were pooled, and diluted two fold with 20 mM HEPES pH 8.3. The 15 sample was then applied with a Super loop onto a Mono S column linked to a FPLC system (Pharmacia, Piscataway, NJ). Elution was achieved with a multilineal gradient (20 mM HEPES pH 8.3 to 20 mM HEPES pH 8.3, 1 M NaCl). After fraction aliquots were tested for bioactivity, the active 20 fractions were pooled and loaded onto a Vydac C₄ HPLC column that had been equilibrated in 0.1% trifluoroacetic acid (TFA), 20% acetonitrile. The column was eluted with a linear gradient of 20 to 45% aqueous acetonitrile. Aliquots for the bioassay were then taken, and the column 25 fractions were stored frozen at -70°C.

Cell proliferation assays

The mitogenic activity of the column fractions and purified samples was determined by using as target cells

adrenal cortex-derived capillary endothelial cells (ACE cells) (22). Stock cultures, maintained in the presence of the DMEM supplemented with 10% CS, 50 μ m/ml gentamicin, and 0.25 μ m/ml Fungizone were passaged weekly on 5 gelatinized tissue culture dishes at a split ratio of 1:10.

For mitogenic assay, cells were seeded in 12 well cluster plates at a density of 5×10^3 cells per well in 1 ml DMEM supplemented with 10% calf serum and antibiotics, 10 as described previously (19). Six hours later, a set of triplicate wells was trypsinized, and cells were counted to determine the plating efficiency. Ten microliter aliquots of the appropriate dilution of each sample, as indicated in the figure legend detailed descriptions below, were then 15 added in triplicate to wells in the dishes on days 0 and 2. After 4 days in culture, the plates were trypsinized, and cell densities were determined with a Coulter counter (Coulter Electronics, Hialeah, FL).

The mitogenic activity of the final purified material 20 was also tested on human umbilical endothelial cells, bovine granulosa cells, adrenal cortex cells, corneal endothelial cells, BHK-21 cells and BALB/MK mouse epidermal keratinocytes. For assaying, cells were seeded at an initial density of 2 or 4×10^4 cells/35-mm dish. Assays 25 were conducted as described for bovine vascular endothelial cells.

Na Dod SO₄/PAGE

Samples were reacted with 250 μ Ci of Na¹²⁵I using the

chloramine T method of iodination (28). After TCA precipitation in the presence of ovalbumin carrier (100 μ g/ml), the 125 I-labelled samples ($2.5-16 \times 10^4$ cpm in 10 μ l) were analyzed by Na Dod SO₄/PAGE, (15% polyacrylamide, ref. 29) under reducing or non reducing conditions. After electrophoresis (5 hr, 20 mAmp) the gels were stained with 0.1% Coomassie blue in 50% trichloroacetic acid for 15 min and destained overnight with 7% acetic acid. Gels were then dried and subjected to autoradiography for 6 to 92 hr.

10 Protein microsequencing

For protein sequencing, approximately 5 μ g (=200 pmol) of protein from the active fractions of the C₄ column were redissolved in 50% trifluoroacetic acid and loaded onto an Applied Biosystems 477A gas-phase protein sequenator.

15 Twelve rounds of Edman degradation were carried out using standard software and chemicals supplied by Applied Biosystems, and identifications of PTH amino acids were made with an automated on-line HPLC column (model 120, Applied Biosystems, Foster City, CA).

20 Growth Factor Isolation and Detection

Preliminary experiments indicated that media conditioned by FS cells contained considerable amounts of mitogenic activity for capillary endothelial cells which could not be neutralized by specific aFGF or bFGF 25 neutralizing polyclonal antibodies. Furthermore when applied to a HS affinity column in 0.6 M NaCl, the majority of the activity was not retained. In contrast, aFGF and bFGF are both retained under similar conditions, and elute

from HS at NaCl concentrations of 1.1 M and 1.6 M, respectively (18,30,31). Since cultured pituitary cells are known to produce various growth factors (32), the possibility existed that factors such as TGF α , EGF and TGF β might also be present in the conditioned medium from FS cells. ACE cells, which do not respond to TGF α , EGF, or PDGF (22) and for which TGF β is growth inhibitory (33,34), were therefore used to follow the purification of the putative novel growth factor.

(NH₄)₂SO₄ precipitation provided a convenient way of reducing the volume of the collected conditioned medium from the FS cells to a level suitable for subsequent chromatography. HSAC, which has been used for the purification of other growth factors (32, 33) provided an efficient purification step. Material not retained by the column was inactive and accounted for 50% of the total protein loaded (fig. 1A). It is likely that the transferrin and insulin components of the cell media were present in the unretained fraction and contributed to the major portion of the proteins. Elution with 0.15 M NaCl yielded a small peak of protein with no bioactivity, while elution with 0.45 M yielded a major peak of protein with 10% of the bioactivity applied to the column. Preliminary experiments had shown that most of the bioactivity could be eluted from HS with 0.6 M NaCl, but that the activity eluted as a broad peak. Therefore, to concentrate the protein peak, elution with 1 M NaCl was carried out. This step gave a relatively tight protein peak in which 90% of

the bioactivity applied on the column was recovered (fig. 1A). Overall, the HS chromatography resulted in a thirty fold purification, estimated by the protein recovered. Since the growth promoting activity in the starting material was variable, possibly due to the presence of inhibitor, the yield in this step could not be determined exactly (see Table I).
5

Table I

Summary of purification of the Folliculo Stellate derived growth factor

Purification steps	Protein(μg)	Maximal Stimulation (ng/ml)	ED50 (ng/ml)	Total activity (units)	Purification (fold)	Yield %
(NH4)2 SO4 ppt	405000	750 ^a	140 ^a	2.9 × 10 ⁶ a	1	—.a
HSAC 1M NaCl pool ^c	13464	100	15	9 × 10 ⁵	30b	100
Bio Gel P-60 c	1477	10	1.5	9.8 × 10 ⁵	300	108
Mono S frac 39-42 ^c	244	8	0.5	4.9 × 10 ⁵	900	54
C ₄ HPLC ^d	15	0.5	0.05	2.3 × 10 ⁵	6923	25

a) The growth promoting activity in the (NH₄)₂ SO₄ was variable, maybe due to the presence of growth inhibitor. Maximal stimulation at saturation was also much lower than that observed for other partially purified fractions (see fig. 6). Therefore the yield was based on total activity present in the first partially purified fraction. (HSAC, 1 M NaCl).

b) The purification in the HSAC step was estimated at 30 fold based on the amount of protein recovered.

c) Protein was estimated by using the Bradford reagent from Bio Rad with BSA as a standard.

d) Protein was estimated by AA analysis

e) The ED 50 was determined as the protein concentration which gave a half stimulation of cell proliferation in the ACE cell assay. It corresponded to one unit of activity.

HS chromatography was followed by gel exclusion chromatography using Bio Gel P-60 (fig. 1B). The bioactivity eluted as a major peak with an apparent MW of 40 to 45 kDa. This step resulted in a further ten fold 5 purification with a recovery of 100% (Table I).

The bioactive fractions from the Bio Gel P-60 column were pooled and applied to a Mono S column (fig. 1C). The bioactive profile of the eluted fractions consisted of two minor peaks of bioactivity eluting respectively of 0.23 M 10 NaCl and 0.28 M NaCl, with a major bioactive peak eluting at 0.33 M NaCl. Analysis of the bioactivity present in the various peaks indicated that the 0.33 M NaCl fractions contained five fold more activity than either the 0.23 M or 0.28 M NaCl peak (data not shown). The Mono S step gave a 15 further three fold increase in specific biological activity over the Bio Gel P-60 step; recovery was 50% (Table I). The two other bioactive peaks accounted for the remainder of the bioactivity.

Final purification of the endothelial cell mitogenic 20 activity was achieved by RP-HPLC with a C₄ Vydac column (fig. 2A), a preparative method suitable for amino acid sequence analysis. Although losses in biological activity were encountered, presumably because of the acid conditions and solvent used, these were not serious enough to prevent 25 the detection of bioactive fractions. All of the bioactivity detected was present in two closely apposed sharp peaks of protein which, when analyzed by Na₂ Dod SO₄/PAGE gave the same single band on the silver stained

gel (Fig. 3). The RP-HPLC step resulted in at least a seven fold increase in specific biological activity with a recovery of 50% (Table I). Fig. 2B illustrates the relative potency of the various fractions at different 5 stages of purification.

When the bioactive 0.23 M and 0.28 M NaCl pooled Mono S fractions were chromatographed under similar conditions on the C₄ column, a biological profile identical to that observed for the 0.33 M pooled Mono S fractions was 10 obtained (not shown). The major portion (90%) of the bioactivity coincided with two closely apposed peaks of proteins which eluted in the same position as those observed with the 0.36 M NaCl Mono S fraction. When 15 analyzed by Na₂ Dod SO₄/PAGE these peaks gave, under reduced condition, a common band at 23 kDa, migrating in the same position as that observed in Fig. 3.

Physical and Biological Characterization of the Growth Factor

The purified factor when run under unreduced conditions 20 had an estimated molecular weight of 46 kDa (Fig. 3). This value is in good agreement with its elution position on the sizing Bio Gel P-60 column run in solvents expected to maintain the native conformation. When run under reduced condition the apparent molecular mass was 23 kDa (Fig. 3). 25 From these data, it appears that the mitogen consists of two polypeptide chains with molecular mass of 23 kDa. Given that a single N terminal sequence was obtained, the dimeric molecule is probably composed of two identical or

at least very homologous chains.

The apparent fuzziness of the 46 kDa band could be interpreted as indicating the presence of a glycoprotein. In order to explore this point the bioactive HSAC fractions were applied on a concanavalin A Sepharose column in 10 mM Tris pH 7.3, 0.05 M NaCl, 5mM MgCl₂. All of the biological activity was retained by the column (not shown). Elution with high salt (0.5 M NaCl) did not elute any significant amount of bioactivity, while elution with 10 mM methylmannoside did result in the recovery of 25% of bioactivity applied on the column. Elution with 0.2 M methylmannoside did not result in further recovery of bioactivity. These results suggest that the factor is a glycoprotein with strong affinity for concanavalin A. However, the poor biological recovery of the factor from that type of affinity chromatography resin makes concanavalin A unsuitable as a step of purification.

The dose response curves for the growth factor depicted in Figs. 2B and 4 illustrate that as little as 25 pg/ml stimulates ACE proliferation. Saturation was observed at 500 pg/ml with an ED₅₀ of 65 pg/ml (Fig. 4B). These values compared favorably with the range of concentrations where bFGF promotes the proliferation of ACE cells (minimal effect at 10 pg/ml, saturation 200 pg/ml, and ED₅₀ at 50 pg/ml, ref. 22 and Fig. 4B). However, the final density of the culture grown in presence of the FS derived growth factor was half that of cultures exposed to optimal concentrations of bFGF. Nevertheless, if one

considers that the MW of the FS cell derived growth factor is 2.5 times that of bFGF, this new factor has essentially the same potency on a molar basis as bFGF. In addition to its ability to stimulate the proliferation of ACE cells the 5 FS derived growth factor stimulated the growth of bovine brain derived capillary endothelial cells (not shown) as well as that of HUE cells (Fig. 4A). These results indicate that the mitogenic effect of the factor is not limited by species variation nor by the origin of the 10 vascular endothelial cells. However, and in contrast with bFGF, the factor is not mitogenic for BHK-21 cells (Fig. 4C), nor is it mitogenic for adrenal cortex cells, corneal endothelial cells, granulosa cells or BALB/MK cells (Fig. 5). Therefore, and in contrast with FGF, this factor seems 15 to have a unique specificity for vascular endothelial cells.

Description of Figures 1-7

Fig. 1. Purification of FSdGF by HSAC, gel exclusion chromatography and Mono S ion exchange chromatography

20 A. Approximately 350 ml of the $(\text{NH}_4)_2 \text{SO}_4$ precipitate fractions derived from 21 liters of FS cell-conditioned medium and dialyzed against 10 mM Tris HCl pH 7.3, 50 mM NaCl, were loaded onto a heparin Sepharose column (1.5 cm x 12 cm, 25 ml bed volume) at a flow rate of 150 ml/hr. The column was then washed with 150 ml of the equilibration buffer (20 mM Tris-HCl pH 7.3, 50 mM NaCl), and the 25 retained proteins (50% of the total protein applied on the column) were eluted with a stepwise application of

increasing NaCl concentrations (0.15 M, 0.45 M, 1 M and 3 M NaCl.). Fraction size was 2 ml, and the flow rate was 60 ml/hr. Chromatography was performed at 4°C and absorbancy was monitored at 280 nm. The histogram and open circles 5 show the relative ability of the different pooled or individual fractions to stimulate the proliferation of low density ACE cell clutures (5×10^3 cells/35mm dish). In the case of the pooled fractions (elute, wash and 0.15 M NaCl), aliquots were diluted ten fold in 0.2% gelatin in 10 PBS and 10 μ l aliquots were bioassayed. In the case of the individual 0.45 M and 1 M NaCl fractions, aliquots were diluted one hundred fold in 0.2% gelatin in PBS, and 10 μ l aliquots were bioassayed. The majority of the biological activity was present in the 1 M NaCl eluate.

15 B. After concentrating the 1 M NaCl HSAC bioactive fractions 126 to 133 to 1 ml in an Amicon YM10 concentrator, the ultrafiltration retentate was applied on a Bio Gel P-60 column (100-200 mesh, 1 x 95 cm) equilibrated and run at 4°C in PBS. The flow rate for 20 development of the column was 6 ml/hr, and 1.45 ml fractions were collected. Absorbancy was monitored at 280 nm. The elution positions of molecular mass markers (in kDa) were as indicated by the arrows. Aliquots of each fraction from the column were diluted 1 to 100 in 0.2% gelatin in PBS, and 10 μ l aliquots were bioassayed in ACE 25 cells in 12 well dishes, as described in Material and Methods. Most of the bioactivity eluted as a single peak with an apparent MW of 40 to 45 kDa.

C. The bioactive fractions 26 and 29 eluted from the Bio Gel P-60 column were pooled and diluted three fold with 20 mM HEPES pH 8.3. Using a 50 ml Super loop, the sample was then applied on a Mono S HR 5/5 column equilibrated in the 20 mM HEPES pH 8.3 room temperature. The column was eluted with a multilinear gradient of NaCl (0 M to 1 M) as follows: 0 M NaCl for 5 min, 0 M NaCl to 0.45 M NaCl in 45 min, 0.45 M NaCl to 1 M NaCl in 15 min, 1 M NaCl for 5 min. Absorbancy was monitored at 280 nm. Flow rate was 1 ml per min and 1 ml fractions were collected. Aliquots of each fraction were diluted 1 to 100 in 0.2% gelatin in PBS, and 10 μ l aliquots were bioassayed on ACE cells in 12 well dishes as described in Materials and Methods. The histograms show the distribution of the biological activity with most of the biological activity eluting in fractions 37 to 40 (0.33 M NaCl). Fractions indicated by the asterisks were pooled and further examined by RP-HPLC using C₄ column.

Fig. 2 Reverse phase HPLC of the Mono S purified and bioactive fractions and comparison of the ability of FS cell conditioned medium at various stages of purification to stimulate the proliferation of low density ACE cell cultures.

A. The active Mono S fractions (fraction 38 to 40; Fig. 3) were loaded onto a Vydac C₄ column (25 x 0.46 cm, 5 μ m particle size, 300 Å pore size) equilibrated in 0.1% (v/v) TFA, 20% acetonitrile. The arrows shown the times of injection. Protein was eluted with a 115 min linear

gradient of 20-45% acetonitrile in 0.1% TFA at a flow rate of 0.6 ml/min, at room temperature. Fractions of 1.5 ml were collected except in the region where the bioactivity was expected to elute; in this region fraction volumes were 5 limited manually to the size of the individual peak fractions. Aliquots of each fraction were diluted 1 to 10 with 0.2% gelatin in PBS and bioassayed as described in material and methods. The histogram shows the distribution 10 of the biological activity. The peak fractions (fractions 25, 26) indicated by the asterisks were used individually for structural studies and further analysis of their biological activity.

B. Low density ACE cell cultures (5×10^3 cells/well) were seeded and their proliferation was measured as 15 described in Material and Methods. Samples tested were $(\text{NH}_4)_2 \text{ SO}_4$ precipitate [Δ]; pool of the HSAC 1 M NaCl fractions [\blacktriangle]; pool of the bioactive Bio Gel P-60 fractions [\bullet]; pool of the bioactive Mono S fractions [\square]; bioactive C_4 fraction [\diamond]. Individual points are the mean of 20 triplicate determinations, and standard deviations were less than 10% of the mean. Control cultures exposed to DMEM supplemented with 10% CS had a final cell density of 1.5×10^4 cell/well.

Fig. 3 $\text{NaDODSO}_4/\text{PAGE}$ of the bioactive fractions purified 25 by C_4 RP-HPLC.

The fractions in each of the three peaks of activity from Mono S ion-exchange chromatography step (eluting at 0.23M, 0.28M, and 0.33M NaCl, respectively; see Fig. 1C)

were pooled, and each pool was further purified by C4 RP-HPLC, as described in Fig. 2A. In each case, the two peaks of activity eluting from the C4 column (see Fig. 2A) were collected and pooled. Samples of the pooled material from 5 each of the three runs of the C4 column were then subjected to electrophoresis on a 15% polyacrylamide gel, under reducing (lanes 3 - 5) or non-reducing (lanes 6 - 8 conditions. The gel was then stained using the silver nitrate stain kit of BioRad. The samples were: lanes 3 and 10 6, 0.23 M pool; lanes 4 and 7, 0.28M pool; lanes 5 and 8, 0.33M pool. The molecular weight markers runs in lanes 1, 2 and 9 were: bovine serum albumin (MW 66,000), ovalbumin (MW 42,700), carbonic anhydrase (MW 31,000), soybean trypsin inhibitor (MW 21,500), and lysozyme (MW 14,500).

15 Fig. 4. Comparison of the ability of pituitary derived bFGF versus FSdGF to stimulate the growth of HUE cells (A), ACE cells (B), and BHK-21 cells (C).

(A) Low density cultures of HUE cells (5×10^3 cells per 1 cm diameter gelatinized well) were exposed to HEPES (25 mM) buffered medium 199 supplemented with 100 $\mu\text{g.ml}$ heparin, 10^{-8}M selenium, 20% FCS and increasing concentrations of either pituitary derived bFGF (O) or FSdGF (●). Heparin was added only once at the time of seeding while both bFGF and FSdGF were added every other day. After 6 days in culture, triplicate wells were trypsinized and cell counted. The final desnity of cultures exposed to 20% FCS alone was 1.5×10^4 cells per well. Standard deviation was less than 10% of the mean.

(B) Low density cultures of ACE cells (5×10^3 cells per 1 cm diameter well) were exposed to DMEM supplemented with 10% CS and increasing concentrations of either pituitary derived bFGF (O) or FSdGF (●) added every other day. After 5 days in culture, triplicate wells were trypsinized and cell counted. The final density of cultures exposed to 10% CS alone was 1.3×10^4 cells per well. Standard deviation was less than 10% of the mean.

(C) Low density cultures of BHK-21 cells (2×10^4 cells per 35 mm gelatinized culture dishes) were exposed to 2 ml of DMEM-F12 (1 to 1 v/v) supplemented with 2.5 μ g/ml Fungizone, 50 μ g/ml gentamicin, 10 μ g/ml transferrin, 5 μ g/ml insulin and increasing concentrations of either pituitary derived bFGF (O) or FSdGF (●). Insulin and transferrin were added only once, bFGF and FSdGF were added every other day. After 4 days in culture triplicate dishes were trypsinized and cell counted. The final density of culture exposed to transferring and insulin alone was 1.38×10^4 cells/plate. Standard deviation was less than 10% of the mean.

Fig. 5 Comparison of the ability of bFGF versus FSdGF to stimulate the proliferation of BCE cells, granulosa cells, Adrenal cortex cells and BALB/MK cells.

2×10^4 BCE cells, granulosa cells, or adrenal cortex cells per 35 mm dishes were seeded in the respective media (DMEM supplemented with 10% CS for BCE and adrenal cortex cells and F-12 medium supplemented with 2.5% CS for granulosa cells). BALB/MK cells were seeded at a density

of 3×10^4 cells per 35 mm dishes in low Ca modified Eagle medium (40) supplemented with 10% FCS. Pituitary derived bFGF (bFGF, 2ng/ml) or FSdGF (5ng/ml) were added every other day. BALB/MK cells were also exposed to aFGF (10ng/ml) since this mitogen is as potent as EGF in promoting their growth. After 6 days in cultures, cells were trypsinized and counted in a Coulter counter. Standard deviation was less than 10% of the mean.

10 Microsequencing Reveals a Unique N-Terminal Amino Acid Sequence

A single amino acid was identified in each of the first 12 cycles, consistent with a homogeneous protein. The yield of the amino terminal residue was 150 picomoles. Unambiguous assignments made for cycles 1 to 12 were as follows: Ala-Pro-Met-Ala-Glu-Gly-Gly-Gln-Lys-Pro-His-Glu. A search of the NBRF database using the FASTP program of Lipman and Pearson (35) found no significant homology between this sequence and any known protein.

20 Sequence Analysis of Tryptic Peptides

12 μ g of FSdGF was reduced in 200 μ l of a solution containing 6M guanidine hydrochloride, 0.5M Tris base pH=8, 1mM EDTA and 10mM dithiothreitol for one hour at 37°C. The reduced protein was alkylated by the addition of solid iodoacetamide to give a final concentration of 25mM and the reaction allowed to proceed for 30 minutes at room temperature. Following the alkylation reaction lysines were modified with succinic anhydride. Four aliquots of 5 μ l each freshly prepared 100mg/ml succinic anhydride in

acetonitrile were added at five minute intervals. After the final addition the protein was desalted by reverse phase HPLC using a Hewlett-Packard 1090L HPLC equipped with a diode array detector and a 0.46x3 cm Brownlee Labs C₄ (BU 300) column. Solvent A was 0.1% TFA in water, solvent B was 0.1% TFA in acetonitrile. The gradient was 20-60% B over 30 minutes at 0.5 ml/min flow rate. The elution profile was monitored at 214 nm and the single protein peak was collected by hand. The chromatogram is shown in Figure 6. After drying on a Speed-Vac (Savant) the protein redissolved in 200 μ l of 100 mM ammonium bicarbonate. 0.4 mg of TPCK treated trypsin was added and the sample was incubated for an additional 2 hours at 37°C. An additional 0.4 μ g of trypsin was added after 2 hours and the sample incubated for an additional 2 hours at 37°C. Peptides were separated on the same HPLC using the same solvents as described above. The column was a 0.21 x 15 cm Vydac C₁₈ (218TP5215) run at 0.25 ml/min. The HPLC program initially flowed for 5 minutes at 10% B to and then ran a linear gradient to 10 to 70% B over 65 minutes. The elution of peptides was monitored at 214 nm and peaks were collected by hand. The chromatogram is shown in Figure 7. For amino acid sequence analysis peaks 7, 15, 16, 24 and 25 were spotted and dried directly on the glass fiber discs used as supports in the protein sequencer. No sequences were obtained from peptides 15 and 16. Peptide 7 gave a mixed sequence with the major sequence H-Ile-X-Pro-His-Gln-Ser-Gln-His-Ile-Gly-Glu-Met-Ser-Ile-Leu-Gln-His-Asn- and the

minor sequence H-X-Val-Leu-Asp/Phe-Val-Val-X-X-Pro-. Peptide 24 gave a single sequence H-Ser-Phe-Cys-Arg-Pro-Ile-Glu-Thr-Leu-Val-Asp-Ile-Phe-Gln-Glu-Tyr-Pro-Asp-Glu-Ile-. Peptide 15 gave a single sequence H-Ser-Phe-Cys-Arg-5 Pro-Ile-Glu-Thr-Leu-Val-Asp-Ile-Phe-Gln-Glu-Tyr-Pro-Asp-/Ile-Glu. Ser 1 of the peptides 24 and 25 corresponds to Ser 23 of the amino terminal sequence shown above so that these sequences can be merged to give the sequence of the first 42 amino acids of the protein.

10 Unambiguous assignments made for cycles 1 to 12 were as follows: Ala-Pro-Met-Ala-Glu-Gly-Gln-Lys-Pro-His-Glu. A search of the NBRF database using the FASTP program of Lipman and Pearson (35) found no significant homology between this sequence and any known protein.

15 Compositions and Uses

FSdGF provided by the invention is useful as a wound healing agent, particularly in applications where it is desired to re-endothelialize vascular tissue, or where the growth of a new capillary bed (angiogenesis) is important.

20 FSdGF can, therefore, be used in the treatment of full-thickness wounds such as dermal ulcers, including the categories of pressure sores, venous stasis ulcers and diabetic ulcers. In addition, FSdGF can be used in the treatment of full-thickness burns and injuries site for a skin graft. In this case, the FSdGF is either applied directly to the site or is used to soak the skin that is being transplanted prior to grafting. In a similar fashion, FSdGF will be used in plastic surgery when the

reconstruction is required following a burn, other trauma or for the cosmetic purposes.

Angiogenesis is also important in keeping wounds clean and non-infected. FSdGF can, therefore, be used in association with general surgery and following the repair of cuts and lacerations. It is particularly useful in the treatment of abdominal wounds where leakage of fecal material increases in the risk of infection. Neovascularization is also key to fracture repair since blood vessels develop at the site of bone injury. Adminstration of FSdGF to the site of a fracture is, therefore, another utility.

In the cases where FSdGF is being used for topical wound healing, as described above, it may be administered by any of the routes described below for the reendothelialization of vascular tissue, or more preferably by a topical means. In these cases, it will be administered as either a solution, gel, cream, ointment or as a dry powder directly to the site of injury. Slow release devices direction FSdGF to the injured site will also be used. In topical applications, FSdGF will be applied at concentration ranging from 50 to 1,000 μ g/ml either in a single application, or in dosing regimens that are daily or every few days for a period of one to several weeks.

FSdGF can be used as a post-operative wound healing agent in balloon angioplasty, a procedure in which vascular endothelial cells are removed, together with atherosclerotic plaques. FSdGF can be applied to vasuclar

endothelial surfaces by systemic or local intravenous application either as intravenous bolus injection or infusions. If desired, the FSdGF can be administered over time using a micrometering pump. Suitable compositions for 5 intravenous administration comprise FSdGF in an amount effective to promote endothelial cell growth and a parenteral carrier material. The FSdGF can be present in the composition over a wide range of concentration, for example, from about 50 $\mu\text{g}/\text{ml}$ to about 1,000 $\mu\text{g}/\text{ml}$ using 10 injections of 3-10 ml per patients. Any of the known parenteral carrier vehicles can be used, such as normal saline or 5-10% dextrose.

FSdGF may also be used to promote endothelialization in vascular graft surgery. In the case of either vascular 15 grafts using transplanted vessels of synthetic material, for example, FSdGF can be applied to the surfaces of the graft and/or at the junctions of the graft and the existing vasculature in order to promote the growth of vascular endothelial cells. For such applications, the FSdGF may be 20 applied intravenously as described above for balloon angioplasty or it may be applied directly to the surfaces of the graft and/or the existing vasculature either before or during surgery. In such cases, it may be desired to apply the FSdGF in a thickened carrier material so that it 25 will adhere to the affected surface. Suitable carrier materials include, for example, 1-5% carbopol. The FSdGF may be present in the carrier over a wide range of concentrations, for example, from about 50 $\mu\text{g}/\text{mg}$ to about

1000 μ g/mg.

FSdGF may also be employed to repair vascular damage following myocardial infarction. The FSdGF is administered intravenously for this purpose, either in individual 5 injections or by micrometering pump over a period of time as described above.

FSdGF may also be used as a growth factor for the in vitro culturing of endothelial cells. For such uses, FSdGF can be added to the cell culture medium at a concentration 10 from about 10 ng/ml to about 10 μ g/ml.

The amino acid sequence of FSdGF will be used to design synthetic oligonucleotide probes for the retrieval of the FSdGF gene. These probes will either be of a mixed sequence based on all possible genetic code choices, or 15 will be of single sequence based on codon choice preferences and other factors. In the first instances, probes based on the amino acid sequence of bovine FSdGF will be used to screen either bovine cDNA libraries made from folliculostellate cells, or bovine genomic libraries. 20 Bovine DNA clones encoding FSdGF thus isolated will be sequenced to determine the complete coding and hence amino acid sequence of bovine FSdGF. The bovine FSdGF clones will then be used as probes to isolate human FSdGF sequences from either cDNA libraries generated from tissues 25 shown to express the factor, or from human genomic libraries. In this way, the complete nucleotide and hence amino acid sequence of human FSdGF can be established.

Discussion

This is the first identification, purification and biological characterization of a novel heparin-binding endothelial cell growth factor (VEGF) from culture media 5 conditioned by pituitary FC. Additional detail is provided in the Examples below.

Results

The media conditioned by FC was found to stimulate the proliferation rate of low-density microvascular endothelial 10 cells. Table 2 summarizes the steps for the purification of the growth promoting activity and the corresponding yield in bioactivity. The mitogenic activity was precipitated by 50% ammonium sulfate and resuspended to a volume suitable for subsequent purification. The H-S step 15 provided an efficient way of further concentrating such activity and also provided a ten fold purification. Approximately 90% of the biological activity was eluted in the presence of 0.9 M NaCl (Fig. 8). The bioactivity was not affected heating the fractions at 65° C for 5 min and 20 was decreased 25-30% following the exposure to 0.1% TFA (pH 2) for two hours.

Table 1

Summary of purification of VEGF from 6 liters of conditioned medium.

Purification step	Protein (ug)	Maximal stimulation (ng/ml)	Purification (fold)	Yield (%)
C.M. *	190,000	2500	1	100
A.S. *	175,000	2500	1	92
H-S *	13,000	250	10	68
R-P 1 ^	25	5	500	6
R-P 2 ^	4	1.2	2000	4

C.M., conditioned medium; A.S., ammonium sulfate precipitate; H-S, heparin-sepharose; R-P 1, reversed phase HPLC step 1; R-P 2, reversed phase HPLC step 2.

* protein concentration was determined by Bio Rad Kit

^ protein concentration was determined by comparing the relative intensities of bands with standards in silver-stained SDS/PAGE.

The most bioactive H-S fractions was applied to a semi preparative C4 reversed phase HPLC column, a method suitable for rapid purification of proteins and peptides. The bioactivity was eluted as a single peak in the presence 5 of about 29% acetonitrile (Fig. 9A). A silver-stained (56) SDS/PAGE gel on the most bioactive fractions revealed the presence of three or four bands. These fractions were further purified by a second reversed phase HPLC step, using an analytical C4 column which was eluted with a 10 gradient of 2-propanol, instead of acetonitrile. A single peak of bioactivity corresponding to a distinct peak in the absorption profile was obtained. (Fig. 9B).

The peak fractions from the second reversed phase step displayed a single band on a silver stained SDS/PAGE, with 15 an apparent M_r of about 23 kDa under reducing conditions (Fig. 10). The intensity of staining of the band was highly correlated to the mitogenic activity across the bioactivity profile. Because previous experiments, using a molecular sieve with a TSK G 3000 SW column (data not 20 shown), suggested a M_r in the range of 40-43 kDa, the possibility that the growth factor in native conditions is a dimer was considered. This was strongly suggested by the finding that the purified material had an apparent M_r of about 45 kDa in a silver stained SDS-PAGE under non- 25 reducing conditions (see Fig. 10).

As illustrated in Fig. 11, the dose response curve for the purified growth factor revealed a half maximal effect on adrenal cortex-derived capillary endothelial cells

proliferation at 150-200 pg/ml and a maximal effect at 1-1.5 ng/ml. These values were derived from protein sequencing and were found to be in good agreement with those obtained by comparing the relative intensities of 5 bands with standards in silver stained SDS/PAGE.

Gas phase microsequencing of the purified material demonstrated unambiguously a single N-terminal amino acid sequence. The first five residues are Ala-Pro-Met-Ala-Glu. Another way to describe the N-terminal amino acid sequence 10 is Ala-Pro-Met-Ala-Glu-Gly-Gly-Gln-Lys-Pro-His-Glu-Val-Val-Lys-Phe-Met-Asp-Val-Tyr-Gln-(Arg)-Ser-Phe-X-Arg-Pro-Ile-Glu-Thr-Leu-(Val)-X-Ile-X-(Gln)-Glu-Tyr-(Pro)- where the amino acids in parenthesis are known with a high degree of 15 certainty and -X- denotes an amino acid of as yet unknown identity. A computer search revealed that such a sequence does not display significant homology to any previously known protein.

The bioactivity of the growth factor was also tested with different cell types. As shown in Figure 12, 20 appreciable activity was observed only in cell types of vascular endothelial origin, such as fetal and adult bovine aortic endothelial cells, bovine brain capillary endothelial cells and human umbilical vein endothelial cells. In contrast, adrenal cortex cells, lens epithelial 25 cells, corneal endothelial cells, BHK-21 fibroblasts and keratinocytes failed to show any significant mitogenic response.

The growth factor was purified by using a combination

of ammonium sulfate precipitation, H-S affinity chromatograph and two reversed phase HPLC steps. Analysis of the purified material by SDS PAGE reveale a M_r of about 45 kDa under non reducing conditions. When the material 5 was analyzed in the presence of 2-mercaptoethanol, a single band with a M_r of 23 kDa was visualized, indicating that the growth factor is a dimer composed of two subunits of identical apparent molecular weight. Microsequencing of the purified material reveals a unique N-terminal amino 10 acid sequence.

The growth factor was heat and acid stable and its p.i., as estimated by chromatofocusing on a Mono P column (data not shown) is about 8.5.

The purified growth factor was able to stimulate the 15 proliferation of vascular endothelial cells at concentrations between 25 pg and 1-1.5 ng/ml. These values, assuming a M_r of 45 kDa, correspond respectively to 0.55 pM and 22-33 pM. Such values are in the same range as those obtained with bFGF (2,56). However, the novel growth 20 factor did not induce any appreciable mitogenic effect on corneal endothelial cells, lens epithelial cells, BHK-21 fibroblasts, adrenal cortex cells, or keratynocytes. In contrast, bFGF and aFGF are both potent mitogens for all of these cell types (2,56).

25 The ability of VEGF to bind heparin may have implications as to its in vivo function and regulation. Heparin sulphates are fundamental components of the extracellular matrix and have been proposed to play a

crucial role in determining contact between target cells and heparin-binding growth factors (16, 57, 58, 59).

The presence of VEGF in pituitary FC indicates a role for these cells in the development, organization and 5 maintenance of a differentiated state of the complex microvasculature of the adenohypophysis.

{ It is presently unknown whether VEGF is expressed in organs other than the pituitary gland. However, considering the fundamental role of vascular endothelial 10 cells growth and angiogenesis in a great variety of normal and pathological proliferations (60), it is expected that the distribution of the growth factor is widespread. With this context, it is of interest that PDGF, EGF, TGF alfa, TGF beta, FGF, NGF, which were initially believed to be 15 restricted to specific cells or tissues, were later found to have a much broader and sometimes ubiquitous distribution (61).

The genes for bFGF and aFGF, the best characterized endothelial cell mitogens, do not code for a conventional 20 signal peptide (17, 43). Accordingly, these growth factors appear to be sequestered inside the cells of origin and apparently do not have direct access to target cells (2, 15, 45). It has been suggested that bFGF may be incorporated into the basement membrane and be subsequently 25 released in a soluble form only when the matrix is degraded following the action of specific enzymes (16). Such a mechanism of release suggests a role for the growth factor mostly or exclusively in events which involve degradation

of the basement membrane or cell lysis, such as organ remodeling, wound healing or neoplasia (60).

In contrast, a soluble endothelial cell growth factor such as VEGF may play a more dynamic role in the 5 physiological regulation of the vascular endothelial cells proliferation, either in the cyclical growth of blood vessels which takes place in organs such as the corpus luteum (62) or in the tonic maintenance of the differentiated stage of the endothelium in the vascular 10 tree.

Unlike bFGF or aFGF, which are active on a very broad spectrum of cells (2,56), VEGF appears to be specific for vascular endothelial cells. VEGF is special therapeutic significance for conditions in which a selective action on 15 the vascular endothelial cells, in the absence of excessive connective tissue proliferation, is desirable, such as diabetic ulcers or traumatic vascular injuries.

Detailed Description of Figures 8 to 12

Figure 8 -- Heparin-sepharose (H-S) bioactivity 20 profile of FC conditioned medium. The medium (6 liters) was concentrated and applied to a H-S which had been preequilibrated in 10 mM Tris/Cl, pH 7.2 containing 50 mM NaCl. The column was washed with the same buffer and then eluted sequentially with 10 mM Tris/Cl, pH 7.2, containing 25 0.15, 0.9 and 3 M NaCl. Aliquots of the collected fractions were diluted 100 fold in 0.2% gelatin in PBS 5 μ l/ml were applied to capillary endothelial cells for bioassay.

Figure 9A and 9B -- Sequential reversed phase HPLC

profiles of endothelial cell mitogenic activity. The most bioactive H-S fractions were applied to a C4 column (10 x 250 mm) preequilibrated with 0.1% TFA/20% acetonitrile (panel A). After the column was washed with 10 ml of 5 equilibration buffer, the sample was eluted with a linear gradient of acetonitrile. Aliquots of each fraction were diluted tenfold with 0.2% gelatin in PBS and 5 μ l/ml were applied to capillary endothelial cells for bioassay. The most bioactive fractions were pooled and applied to a C4 10 column (4.6 x 250 mm) which had been preequilibrated with 0.1% TFA/20% 2-propanol (panel B). After washing the column with 3 ml of equilibration buffer, the sample was eluted with a linear gradient of 2-propanol. Aliquots of fractions were tested for bioactivity.

15 Figure 10 -- NaDODSO₄/PAGE analysis of most bioactive fraction from chromatogram shown in Fig. 9B. Two 50 μ l aliquots of such fraction were dried in a speed vac and redissolved in sample buffer containing (+) or not (-) 2,5% 2-mercaptoethanol. The samples were heat-denatured 20 and electrophoresed in a 12.5% PAGE which was subsequently silver stained. The molecular weight markers are: phosphorylase B, 97,400; bovine serum albumin, 66,200; ovalbumin, 43,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400.

25 Figure 11 -- Dose-responsive growth of adrenal cortex derived capillary endothelial in the presence of purified VEGF. Cells were seeded at the density of 1 x 10⁴/well in 12 well plates. The indicated amounts of VEGF were added

a few hours after plating in 5 μ l/ml aliquots. After five days, cells were counted in a Coulter counter. The results shown represent mean values of three separate experiments conducted in duplicate. Duplicates in each experiment 5 varied less than 10%.

Figure 12 -- Effects of VEGF on the growth of different cell types. CEC, corneal endothelial cells; BAC, bovine adrenal cortex cells; KTC, keratynocytes; LEC, lens epithelial cells; BHK-21, baby hamster kidney cells, clone 10 21; ACC, adrenal cortex capillary endothelial cells; BBC, bovine brain capillary endothelial cells; HUVE, human umbilical vein endothelial cells; FBAE, fetal bovine aortic endothelial cells, ABAE, adult bovine aortic endothelial cell. Cells were seeded in their respective growth media, 15 incubated with a maximal concentration of VEGF and counted after 4 or 5 days. Results are expressed as a percent of appropriate control.

The following Examples are provided to be illustrative and exemplary only. They are not to be construed as being 20 limiting in any way.

Reagents -- Tissue culture media and reagents were obtained from Gibco (Grand Island, N.Y.). Acetonitrile and 2-propanol were purchased from Fisher Sci. (Fair Lawn, NJ). Heparin-sepharose (H-S) was obtained from Pharmacia 25 (Piscataway, N.J.). Vydac HPLC columns were from The Separation Group (Hesperia, CA). Molecular weight markers for PAGE and protein determination kit were from Bio Rad Labs (Richmond, CA). Tissue culture plates were purchased

from Costar, except for large scale Nunc plates (24.5 x 24.5 cm), which were Applied Sci. (San Francisco, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO) or Applied Biosystems (Foster City, CA).

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EXAMPLE A

Purpose of this Example was the determination of the molecular weight of the endothelial cell growth factor secreted in the medium by follicular cells.

Confluent cultures of follicular cells were incubated 10 for three days in a serum-free medium consisting of low glucose Dulbecco's modified Eagle's medium supplemented with transferrin (10 μ l/ml), insulin (5 μ g/ml), 2 mM glutamine and antibiotics. The conditioned medium (CM) (150 ml) was then collected centrifuged (10000 xg, 15 min. 15 4°C) in order to remove cell debris, and then applied to a Heparin-Sepharose column which had been preequilibrated with 10 mM Tris/Cl, pH 7.0. The column was then sequentially eluted with 10 mM Tris/Cl, pH 7.0 containing 0.6, 1 and 3 M NaCl. The flow rate was 21 ml/h. Fractions 20 of 700 μ l were collected and aliquots were tested for bioactivity on adrenal cortex-derived microvascular endothelial cells. The majority of the bioactivity was eluted in the presence of 0.6 M NaCl. This chromatographic behavior is different from that of aFGF or bFGF, which are known to elute, respectively, in the presence of 0.9-1.1 M NaCl and 1.8-2.2 M NaCl.

The most bioactive 0.6 NaCl fractions were pooled and further examined for determination of the molecular weight

of growth factor activity. A standard 12.5% polyacrilamide SDS slab gel was prepared. Ten percent glycerol and 2% SDS were added to the pooled fractions. Fifty per cent of the sample was treated with 2.5% 2-mercaptoethanol. The remainder 50% was not exposed to 2-mercaptoethanol or other reducing agents. The samples and prestained molecular weight markers were then incubated for 3 min at 37°C and electrophoresed overnight at a current of 10 mA. When the electrophoresis was completed, the gel was briefly rinsed in PBS and the distance of the molecular weight markers from the top of the gel was immediately measured. Twenty-one half centimeter horizontal slices were then cut with a razor blade both from the lanes run under reducing and those run under non-reducing conditions. Slices were then washed twice with 1 ml of PBS and then shaken overnight at 4°C in individual tubes containing 500 μ l of 0.2% gelatin in PBS for elution of the biological activity. The gel slices were then removed from the tubes, which were then centrifuged in order to remove particulate material. The supernatants were transferred to new tubes. Twenty microliter aliquots from each fraction were tested for biological activity on endothelial cells.

A single peak of bioactivity was observed in the group not exposed to 2-mercaptoethanol. The apparent molecular weight, as assessed by comparing the position of the molecular weight markers to that of the slices, was about 43,000. No bioactivity was recovered from slices exposed to 2-mercaptoethanol.

These results gave a good assessment of the molecular weight of the growth factor, which has been confirmed with the molecule purified to homogeneity, and also indicated that its activity is abolished by reducing agents.

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EXAMPLE 1CULTURE OF FOLLICULAR CELLS AND MEDIA COLLECTION

Primary cultures of bovine pituitary FC were established as previously described (20,41). In one embodiment in the culturing the 20% fetal bovine serum in reference 20 was reduced to 10%. Concentrations of 5 to 20% should be effective. Also no DNAase is used. All other components are the same. At confluence, cells were passaged into large scale tissue culture plates in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics. Shortly after reaching confluence the cultures were extensively washed with PBS in order to remove serum components. The cells were then incubated in a serum-free medium consisting of DMEM plus transferrin (10 μ l/ml), insulin (5 μ g/ml), selenium (10^{-8} M), 2 mM glutamine and antibiotics. After three or four days, the medium was collected and replaced with fresh serum free medium. The collected medium was centrifuged (1000 g, 15 min. at 4°C) and stored at -70°C. The conditioned medium (CM) was then collected every three or four days for up to six weeks.

EXAMPLE 2CONCENTRATION OF CONDITIONED MEDIUM

Four to six liter batches of conditioned medium (CM) were subjected to ammonium sulfate precipitation. Ammonium sulfate (500 g/L) was added under constant stirring, until the salt was completely in solution. After 8-12 hours in the cold room, the material was centrifuged (20,000 xg, 45 min at 4°C). The supernatant was discarded and the pellet was resuspended with 10 mM Tris/Cl, pH 7.2, 50 mM NaCl and dialyzed at 4°C against the same buffer for 8-12 h. The final volume was 50-60 fold less than the original.

Alternatively, the CM is concentrated using ultrafiltration using an Amecon stir cell (2.5 liter unit) using a membrane having a molecular weight cut off of 15 10,000 daltons with similar results.

EXAMPLE 3HEPARIN-SEPHAROSE AFFINITY CHROMATOGRAPHY

The concentrated CM was applied to a H-S column (14) (10 ml) preequilibrated with 10 mM Tris/Cl, pH 7.2, 50 mM NaCl. The column was then washed with the same buffer until the absorbance at 280 nm was negligible and then eluted stepwise with 10mM Tris/Cl pH 7.2 containing 0.15, 0.9 and 3 M NaCl. The flow rate was 1.5 ml/min. Fractions of 1.5 ml were collected and aliquots, diluted with 0.2 % gelatin in PBS, were tested for mitogenic activity on endothelial cells.

EXAMPLE 4REVERSE PHASE HPLC

(a) The most bioactive H-S fractions (0.9 M NaCl pool) were diluted fourfold with 0.1% trifluoroacetic acid (TFA) in water and applied to a Vydac C4 HPLC column (10 x 250 mm) preequilibrated in 0.1 TFA/20% acetonitrile. The column was eluted with a linear gradient of acetonitrile (20-45% in 115 min) at a flow rate of 2 ml/min. The absorbance was monitored at 21 nm. Fractions of 2 ml were diluted in 0.2% gelatin in PBS for assay on endothelial cells.

(b) The most bioactive fractions were pooled, diluted two fold in 0.1% TFA water and applied to a Vydac C4 HPLC column (4.6 x 250 mm) preequilibrated in 0.1% TFA/20% 2-propanol. The column was eluted with a linear gradient of 2-propanol (20-45% in 113 min). The flow rate was 0.6 ml/min. Aliquots of fractions were diluted for bioassays. The remainder of fractions were dried in a Speed-Va for SDS/PAGE (29) and structural analysis.

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EXAMPLE 5BIOASSAYS

Bovine adrenal cortex or brain-derived capillary endothelial cells, adult or fetal bovine aortic endothelial cells, human umbilical vein endothelial cells, bovine corneal endothelial cells, adrenal cortex cells, lens epithelial cells, BHK-21 fibroblasts and human keratynocytes were cultured and maintained as previously described (17,47,48,50,51,52,26,53). For bioassay, cells

were seeded in the presence of their respective growth media at the density of 2×10^4 /35 mm dish or 1×10^4 /well in 12 multiwell plates. Fractions were added to cells in 5 $\mu\text{l}/\text{ml}$ aliquots. After 4 or 5 days, cells were 5 dissociated by exposure to trypsin and counted in a Coulter counter.

EXAMPLE 6

PROTEIN MICROSEQUENCING

10 Approximately 20 pmol of protein from the most bioactive fractions obtained from the second C4 step were applied directly to a gas phase protein sequenator Model 470A (Applied Biosystems). Edman degradation cycles were carried out and identification of amino acid derivatives was made by an on line HPLC column (54).

15 While only a few embodiments of the invention have been shown and described herein, it will be come apparent to those skilled in the art that various modifications and changes can be made in the present invention to the novel endothelial cell growth factor, its methods of isolation, 20 manufacture using recombinant DNA methods and its uses in therapy (wound healing) without departing from the spirit and scope of the present invention. All such modification and changes coming within the scope of the appended claims are intended to be carried out thereby.